

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Luet L. Wong et al.

Serial No. 10/505,341

Filed: November 8, 2004

For: ELECTROCHEMICAL DETECTION OF NADH OR NADPH

DECLARATION

I, Luet L. Wong, do hereby declare and state as follows:

1. I am a university lecturer in chemistry at Oxford University and consultant at Oxford Biosensors. My research interests include the enzymology and genetics of redox active enzymes, electron transfer between proteins and small molecules, electrodes and other proteins, biotransformation and biocatalysis. At Oxford Biosensors, I am responsible for providing expert advice to the scientific and technical teams in research and development.
2. I am an inventor of US Patent Application No. 10/505,341 (USSN 10/505,341) and am therefore familiar with its subject-matter. I have read the Official Action dated 29 April 2008 on USSN 10/505,341. I understand that the US Patent Office Examiner has rejected the claims in USSN 10/505,341 to electrochemical methods and cells as being anticipated by and/or obvious from the prior art disclosures of Cosnier *et al* (Electroanalysis, 1997, 9(9), p685-688) and Bu *et al* (Analytical Chemistry, 1998, 70(20), p4320-4325).
3. I have read and understood Cosnier *et al* and Bu *et al*. I have also read and understood the amended claims to be filed in response to the Official Action dated 29 April 2008. I have been asked to comment on the differences between the subject-matter of these claims and the electrochemical systems disclosed in Cosnier *et al* and

Bu *et al.* References in this Declaration to the claims of USSN 10/505,341 are therefore to the new claims filed in response to the Official Action dated 29 April 2008.

4. The invention set out in USSN 10/505,341 relates to electrochemical methods for monitoring the activity of a dehydrogenase enzyme or for measuring the amount of a substrate for such an enzyme in a sample. A buffered solution is provided, which comprises the substrate-containing sample and:

- (a) a dehydrogenase enzyme, which uses NAD^+ or NADP^+ as a co-factor, for converting the substrate into its product(s);
- (b) NAD^+ or NADP^+ co-factor;
- (c) a NADH or NADPH reductase; and
- (d) a redox active agent;

The target substrate is rapidly oxidised, producing NADH or NADPH, which rapidly reduces the reductase. The reductase in turn rapidly reduces the redox active agent. The quantity of reduced redox active agent is measured by electrochemical means.

5. USSN 10/505,341 also claims an electrochemical cell suitable for carrying out the methods of the invention. This cell contains a mixture of enzymes and redox agent, comprising the dehydrogenase enzyme, the NAD^+ or NADP^+ co-factor, the NADH or NADPH reductase and the redox active agent. In one embodiment, this mixture is present as a buffered solution. In a second embodiment, the mixture is provided in form obtainable by drying such a buffered solution. In this second embodiment, the mixture can be redissolved simply by addition of a liquid sample.

6. USSN 10/505,341 accordingly claims systems in which the enzymes and redox active agent are not immobilized. Rather, when the methods of the invention are carried out the enzymes and the redox active agent are present in a buffered solution that also contains the substrate.

7. Cosnier *et al* teaches two distinct bioelectrode systems, which are both different from that claimed in USSN 10/505,341. The first bioelectrode system described in Cosnier *et al* comprises a "poly-1-Fre-electrode", which is an electrode on which flavin reductase ("Fre") has been immobilized by polymerization of a

mixture of the reductase and a specific amphiphilic pyrrole monomer adsorbed on the electrode surface (see section 2.2 at page 686 of Cosnier *et al*). This electrode is used in assays for detection of NAD(P)H (see Figure 1 of Cosnier *et al*). The second bioelectrode system described in Cosnier *et al* comprises a "poly-1-Fre-LDH-electrode", which is an electrode on which both flavin reductase and lactate dehydrogenase ("LDH") have been immobilized using an analogous procedure (see page 687, column 1, second paragraph of Cosnier *et al*). This electrode is used in assays for detection of a lactate substrate present in a phosphate buffer solution also comprising riboflavin and NAD⁺ (see Figure 4 of Cosnier *et al*).

8. I understand therefore that Cosnier *et al* teaches a method for detecting a lactate substrate using lactate dehydrogenase, NADH, flavin reductase and riboflavin. However, the clear teaching of Cosnier *et al* is that this method requires an electrochemical system in which the dehydrogenase enzyme and the reductase have been co-immobilized on the electrode surface.

9. Bu *et al* also teaches an electrochemical system for the detection of NADH or NADPH. It explains in the abstract that the sensors were obtained by entrapping (i.e., immobilizing) either lipoamide dehydrogenase ("LD") or glutathione reductase ("GR") in a redox gel formed by the copolymerization of vinylferrocene with acrylamide and N,N'-methylenebisacrylamide (see lines 1-5 of the abstract). The redox gel was "secured on the surface" of a carbon paste electrode with a dialysis membrane (see lines 7 to 9 of the abstract).

10. The redox gel on the surface of the Bu *et al* electrode will therefore contain ferrocene moieties. These will act as an immobilized redox active agent for mediating the electron transfer reactions of the lipoamide dehydrogenase or glutathione reductase. Bu *et al* therefore also teaches that immobilization of the redox active agent and the reductase species is necessary to detect NADH or NADPH by an electrochemical assay method.

11. If I were reading Cosnier *et al* and Bu *et al* prior to making the invention set out in USSN 10/505,341, I would have understood from the consistent teaching of these documents that immobilization of the assay reagents onto an electrode surface is

essential in order to obtain a workable dehydrogenase-based biosensor.

Immobilization of assay reagents onto an electrode surface is generally effected when it is necessary to hold the reagents in close proximity to the electrode to ensure an adequate reaction and/or to protect reagent enzymes against degradation. Neither Cosnier *et al* nor Bu *et al* make any reference to assay methods in which all of the assay reagents are present in solution. I would therefore have understood from these documents (a) that it is necessary to immobilize at least the reductase, and in some cases the redox agent as well, onto the electrode surface in order to achieve a workable assay system based on a substrate / dehydrogenase / NAD(P)H / reductase / redox agent cascade, and (b) that an assay method along such lines in which the reagents are not immobilized, but are instead present in a buffered solution, would not be feasible.

12. On the basis of the teachings of Cosnier *et al* and Bu *et al*, I therefore believe that the success of the assay methods claimed in USSN 10/505,341 is surprising. These assay methods also have a number of important technical advantages. In particular, there are clearly significant advantages in terms of reduced preparation costs, since there is no longer any need to prepare special, modified electrodes by complex and time-consuming procedures. Furthermore, the concentration ranges over which the assay methods of USSN 10/505,341 are capable of quantitating a substrate are dramatically improved.

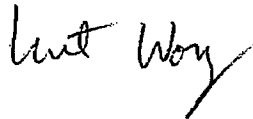
13. In this latter regard, a serious problem with the immobilized systems described in Cosnier *et al* and Bu *et al* is the rather small range over which the electrochemical response is linearly dependent on the concentration of the test species. Thus, in column 2 of page 686 of Cosnier *et al*, the first bioelectrode system is shown to be capable of quantitating NADH up to a concentration of 100 μM and NADPH up to concentration of 145 μM , respectively (see last line before Figure 2). Furthermore, it is taught at page 688, column 1, lines 3 to 5 that a linear amperometric response of the second biosensor to lactate substrate can only be achieved up to 23 μM . In Bu *et al*, which teaches only detection of NAD(P)H, the maximum achievable linear response range is said to be up to 4 mM (page 4323, final paragraph of column 1).

14. In contrast, the assay methods claimed in USSN 10/505,341, which do not involve reagent immobilization on the working electrode, are capable of quantitating substrate up to much higher concentrations. The Experimental Report attached as an Annex to this Declaration details experiments performed under my supervision. It shows clearly that a linear response can be obtained to NADH concentrations of up to at least 10 mM. This is at least twice the maximum achieved by Bu *et al* and an order of magnitude above the maximum achieved by Cosnier *et al*.

15. In conclusion, I would understand both Cosnier *et al* and Bu *et al* to be teaching that it is essential to immobilize at least the reductase on the surface of the working electrode in order to obtain a workable biosensor based on a substrate / dehydrogenase / NAD(P)H / reductase / redox reagent cascade. Further, the arrangement claimed in USSN 10/505,341, in which all assay reagents are present in solution when the assay is carried out, enables important technical advantages as compared with the systems taught in Cosnier *et al* and Bu *et al*.

16. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements are made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this declaration, the patent application, or any patents issuing thereon.

Signed



This 20th Day of October 2008.

ANNEX TO DECLARATION FROM LUET L. WONG

Enzyme mixture

Solution 1 was prepared containing 0.1 M Tris (pH 9.0), 10% sodium taurocholate (NaTC) and 160 mM $\text{Ru}(\text{NH}_3)_6\text{Cl}_3$. Solution 2 was prepared containing 8.3 mg/ml putidaredoxin reductase (PdR) using solution 1. Solution 3 was prepared containing 40 mM nicotinamide adenine (NADH) using solution 1. Dilutions of solution 3 were made using solution 1, to obtain 5, 10 and 20 mM NADH solutions. NADH solutions were stored on ice and used immediately after preparation.

Wet testing protocol

For testing, 5 μL of solution 2 was mixed with 5 μL of NADH solution, and 5 μL of the mix was applied to an electrode strip (note that the wells were formed by punching). The Autolab program was started 30 seconds after initial addition of solution 2 to NADH solution.

The final mix contained:

0.1 M Tris (pH 9.0)

10% NaTC

80 mM $\text{Ru}(\text{NH}_3)_6\text{Cl}_3$

4.15 mg/ml PdR

x mM NADH, where NADH concentration were:

2.5 mM NADH

5 mM NADH

10 mM NADH

Testing protocol

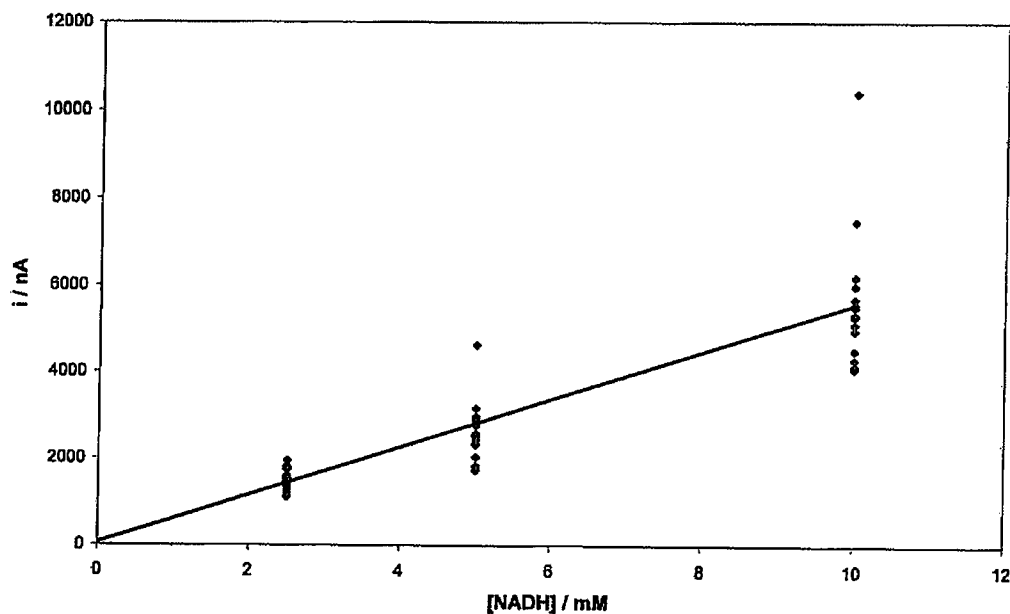
On the addition of 5 μL of plasma, the chronoamperometry test was initiated using a multiplexer (MX452, Sternhagen design) attached to an Autolab (PGSTAT 12). The oxidation current was measured at 0.15 V at one time point (60 seconds), with a reduction current measured at -0.45 V at the final time point (74 seconds). The transient current was measured for 1 second. Each sample was tested in quadruplicate (12 electrochemical cells).

Analysis

The output from the GPES software was analysed using the DataAnal 2 programme.

Results

The sensor responses were plotted vs. NADH concentrations:



All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this declaration, the patent application, or any patents issuing thereon.

Signed *Lucretia Woy*

This 20th Day of October 2008.